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A Next-Generation Sequencing Approach to Characterize the Impacts of Land-based Sources of Pollution on the Microbiota of Southeast Florida Coral Reefs

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ABSTRACT

Coral reefs are dynamic ecosystems known for decades to be endangered due, in large part, to anthropogenic impacts from land-based sources of pollution (LBSP). In this study, we utilized an Illumina-based next-generation sequencing approach to characterize prokaryotic and fungal communities from samples collected off the southeast coast of Florida. Water samples from coastal inlet discharges, oceanic outfalls of municipal wastewater treatment plants, treated wastewater effluent before discharge, open ocean samples, and coral tissue samples (mucus and polyps) were characterized to determine relationships between microbial communities in these matrices and those in reef water and coral tissues. Significant differences in microbial communities were noted among all sample types but varied between sampling area. Contamination from outfalls was found to be the greatest potential source of LBSP influencing native microbial community structure among all reef samples, although pollution from inlets was also noted. Notably, reef water and coral tissue communities were found to be more greatly impacted by LBSP at southern reefs, which also experienced the most degradation during the course of the study. Results of this study provide new insights into how microbial communities from LBSP can impact coral reefs in Southeast Florida and suggest that wastewater outfalls may have a greater influence on the microbial diversity and structure of these reef communities than do contaminants carried in runoff although the influence of runoff and coastal inlet discharge on coral reefs are still substantial.

21 **IMPORTANCE**

22 Coral reefs are known to be endangered due to sewage discharge and to runoff of nutrients,
23 pesticides, and other substances associated with anthropogenic activity. Here we used next-
24 generation sequencing to characterize the microbial communities of potential contaminant
25 sources in order to determine how environmental discharges of microbiota and their genetic
26 material may influence the microbiomes of coral reef communities and coastal receiving waters.
27 Runoff delivered through inlet discharges impacted coral microbial communities, but impacts
28 from oceanic outfalls carrying treated wastewater were greater. Geographic differences in the
29 degree of impact suggest that coral microbiomes may be influenced by the microbiological
30 quality of treated wastewater.

INTRODUCTION

Coral reefs are highly diverse ecosystems that play crucial roles in maintaining marine biodiversity and productivity, coastal protection, and serve as a source of food and recreation (1). Corals exist as holobionts (2), composed of a coral polyp, endosymbiotic zooxanthellae (*Symbiodinium* spp.) (3), bacteria (4), fungi (5), archaea (6), and viruses (7). The interaction of all of the constituents of the coral microbiome have recently been described as dynamic, changing in response to seasonal variations and with disease state (8). Due to global climate change and other anthropogenic impacts on the coral microbiome (1, 9), coral reefs have been recognized as endangered ecosystems for the last several decades (10). Estimates suggest that 20% of coral reefs globally are already lost, while approximately 24% face imminent risk and another 26% may be facing severe damage (1).

A core microbiome among corals is defined functionally rather than based on the presence of specific taxa (4, 11–13), similar to what is found in humans (14). The coral microbiome has recently been implicated in the onset of reef diseases, where stresses on the microbiome (*e.g.* elevated temperature) disturb normal host resistance and/or restriction from other members of the microbiome. Consequently, this allows overgrowth of typically commensal taxa and various opportunistic pathogens (11, 15). Variation in the coral microbiome has also been shown to follow seasonal dynamics (8, 12, 16), with temperature having a more significant impact on community composition than does the disease state (8, 12). However, the functional states of the coral microbiome show some plasticity, adapting to geographical differences, as well as nutrient availability (13).

52 Anthropogenic impacts, primarily in the form of terrestrial runoff, also contribute to
53 nutrient loading, sediment deposition, and the transport of pesticides, pharmaceuticals, and other
54 harmful chemicals to nearby coral reefs, stressing coral communities (17, 18). The parameters
55 influenced by these land-based sources of pollution (LBSP) have been shown to be dependent on
56 the surrounding land cover (19). Not surprisingly, the concentration of pollutants and the extent
57 of discharge from LBSP are directly related to rain events that increase river flows (16, 20, 21).
58 Importantly, LBSP and their associated changes in water chemistry also influence proximate
59 marine communities (16, 22). During periods of high flow (*i.e.* during rain events), the
60 abundances of *Burkholderiales* and *Sphingobacteriales*, common to river samples, increased in
61 marine waters and were correlated with changes in physicochemical parameters (16).

62 It was previously suggested that threats to corals can be broadly divided into global
63 threats, representing climatic or ecological phenomena that are difficult for resource managers to
64 control, and local threats, that refer to specific anthropogenic activities that can be directly
65 modulated and regulated (1). Probiotic approaches, such as phage therapy, bioaugmentation, or
66 adaptation of the commensal coral microbiota have been suggested as means to combat more
67 global threats (11, 23, 24), but the ethics and potential consequences of these actions must be
68 carefully considered. Locally, nutrient runoff, sewage discharge, coastal construction, and
69 overfishing represent more manageable stressors to protect coral communities (reviewed in 1).
70 While the negative consequences of these actions have been generally understood for decades,
71 their impacts on the coral microbiome have only recently received attention (16, 22).

72 In this study, we used a next-generation sequencing approach to characterize the
73 microbial communities from LBSP (coastal inlets, oceanic outfalls from wastewater treatment
74 plants, and wastewater treatment effluent), coral reef waters, and coral tissues (mucus and

polyps) among coral reefs off the southeastern coast of Florida, offshore of Miami-Dade and Broward Counties. We hypothesized that the impacts of various LBSP on microbial (prokaryotic and fungal) communities in nearby coral reefs and tissue samples would vary as a result of demographic and hydrological differences associated with sampling sites, and that the relative degree of source impacts could be determined based on exchange between microbial communities from LBSP and reef waters or coral tissues. Here, potential contributions from LBSP were evaluated using SourceTracker analysis (31–33) which utilizes a Bayesian approach to assign operational taxonomic units (OTUs) from sink communities to sources. This work is a component of a larger Florida Area Coastal Environment (FACE) program study [http://www.aoml.noaa.gov/themes/CoastalRegional/projects/FACE/faceweb.htm]. The goals of this program are to investigate nutrient concentration and transport from LBSPs, perform coral benthic surveys of coral cover and health, and monitor microbiological water quality of the Southeast Florida sentinel coral reef sites used in this study. The results of these other source tracking investigations from the broader FACE study are primarily reported elsewhere (25, 26), and the work we report here focuses on the characterization of microbial communities and potential sources of contamination using next-generation sequencing.

MATERIALS AND METHODS

Sample collection

Water and coral samples were collected during 2014-2015 from three coastal Inlets (Port of Miami, Baker's Haulover, Port Everglades), two treated wastewater effluents (Miami Central and Miami North), two surface boil expressions of oceanic outfalls from treated wastewater

97 (Miami Central and Miami North), 16 coral reef water sites (four reefs with three sites each for
98 surface water, four reefs with site each for bottom), 12 coral polyp tissue extracts (four reefs with
99 three sites each) and 12 coral mucus extracts (Figure 1, described in detail in Supplementary
100 Methods). Water samples were collected on 12 bimonthly, 2-day sampling cruises as part of the
101 FACE program Numeric Nutrient Criteria (NNC) cruises aboard the 41-foot National Oceanic
102 Atmospheric Administration (NOAA) research vessel *Hildebrand* (described in detail in
103 Supplementary Methods). Coral tissue (polyps) and mucus samples were collected on a quarterly
104 basis from 2014-2015 from two different coral species (*Porites asteroids* and *Siderastrea*
105 *siderea*) from the same three reef sites at each of the same four sentinel reefs (Emerald, Pillars,
106 Barracuda, and Oakland), described in detail in Supplementary Methods.

107

108 **Sample processing and DNA extraction**

109 Water samples were processed and extracted as previously described (25). In brief, 1 L
110 water samples were aseptically filtered through 0.2 µm pore-sized, 47 mm diameter, sterile
111 mixed cellulose ester membrane filters (EMD Millipore, Billerica, MA, USA). Filters were
112 aseptically transferred with sterile forceps to Lysing Matrix "A" bead-beat tubes (MP
113 Biomedicals, LLC, Santa Ana, CA, USA), and stored frozen at -80°C until DNA extraction.
114 DNA was extracted from filters using the FastDNA spin kit (MP Biomedicals) as per
115 manufacturer's directions, and stored frozen at -80 °C until further analysis.

116 Coral mucus samples (60 mL) were aseptically filtered onto polycarbonate membrane
117 filters (0.2 µm pore size, 47mm diameter), then aseptically transferred to Lysing Matrix "E"
118 tubes (MP Biomedicals), then extracted with the FastDNA Spin Kit for Soil (MP Biomedicals),

using the Lysing Matrix "E" as per manufacturer's directions. The coral polyp tissues were preserved in 95% ethanol and collected by aseptically filtering he material onto sterile polycarbonate membrane filters (0.2 µm pore size, 47mm diameter). Large pieces of tissue were transferred from the filter into Lysing Matrix "E" tubes (MP Biomedicals) using sterile forceps. Then filter was rolled using sterile forceps and aseptically placed into the same Lysing Matrix "E" tube with the rest of its tissue using sterile forceps. DNA was then extracted using the FastDNA Spin Kit for Soil (MP Biomedicals).

Next-generation Illumina sequencing

Prokaryotic sequencing was performed using the 515F/806R primer set targeting the V4 region (27) and fungal sequencing was performed using the ITS1F/ITS2 primer set targeting the ITS1 region (28). Amplification and sequencing was performed using the dual-index method by the University of Minnesota Genomics Center (Minneapolis, MN, USA)(29), and each sample plate included a sterile water negative control that was carried through amplification and sequencing. Sequencing was performed on the Illumina HiSeq2500 and MiSeq platforms, and results have been shown to be comparable across platforms (27).

Bioinformatics

All sequence processing, unless otherwise noted, was performed using mothur software ver. 1.34.0 (30). Prokaryotic sequence data were trimmed to the first 160 nucleotides (nt) and paired-end joined using fastq-join software (31). Sequences were trimmed for quality as

140 described previously for V5-V6 data (32). Global alignment was performed against the SILVA
141 database ver. 119 (33), sequences were subjected to a 2% pre-clustering step to remove sequence
142 errors (34), and chimeric sequences were identified and removed using UCHIME (35).
143 Operational taxonomic units were assigned at ≥97% identity by complete-linkage clustering.
144 Taxonomic assignments were made against Ribosomal Database Project ver.14 (36), at a
145 bootstrap cutoff of 60% as described previously (37). Fungal sequence data were trimmed to the
146 first 150 nt and processed in the same way as prokaryotic data, with the exception that sequences
147 with homopolymers >9 nt were removed, and fungal assignments were made using the UNITE
148 database ver. 6 (38). For statistical comparisons the prokaryotic dataset was rarefied by random
149 subsample to 35,000 sequence reads per sample, prior to OTU calling, and the fungal dataset was
150 rarefied to 10,000 reads per sample (39).

151 To evaluate potential contributions from LBSP, the software SourceTracker ver. 0.9.8
152 was used to analyze the sequencing data with the default parameters (40). This software utilizes a
153 Bayesian algorithm to identify OTUs from source communities found in sink communities at
154 rarefaction to 1,000 sequence reads. The microbial communities from (1) treated wastewater
155 effluent before oceanic discharge, (2) treated wastewater oceanic outfalls (at the surface), coastal
156 inlet discharge waters, and open ocean surface water communities were designated as the sources
157 for analysis by this SourceTracker algorithm. The open ocean background communities were
158 included as sources to reduce noise associated with outfall samples, due to high community
159 similarity between these sample types.

160

161 **Statistical analysis**

Since samples were collected on adjacent sampling dates, representing a northern and southern sampling area, analyses were performed separately with respect to sampling region to take into account temporal and geographic differences among samples. Statistics were calculated using mothur, unless otherwise stated. The Shannon index and abundance-based coverage estimate (ACE) parameter were calculated as parametric and non-parametric measures of diversity. Differences in beta diversity were evaluated using analysis of similarity (ANOSIM) (41) based on Bray-Curtis dissimilarity distances (42). Non-parametric differences in OTU abundances were evaluated using the Kruskal-Wallis test (43). Ordination of Bray-Curtis dissimilarities was performed using principal coordinate analysis (PCoA) (44), and significance of clustering was evaluated using analysis of molecular variance (AMOVA) (45). ANOVA with Tukey's *post-hoc* test, Spearman correlation, binary logistic regression, and redundancy analyses were performed using XLSTAT software ver. 2015.1.01 (Addinsoft, Belmont, MA, USA). All statistics were evaluated at $\alpha = 0.05$. For redundancy analysis (RDA), the 15 most abundant families were included in the analysis, normalized as percent of total sequence reads per sample. Physicochemical variables were transformed to a number between 0 and 1 in XLSTAT for RDA.

Accession numbers

Sequence data are deposited in the Sequence Read Archive of the National Center for Biotechnology Information under BioProject accession number SRP076111.

RESULTS

Monitoring nutrients and microbiological qualities of water

Water samples (*i.e.* inlet, reef water, open ocean water, and outfall samples) differed significantly in all physiochemical parameters measured (Table 1), except for temperature (P = 0.114) and dissolved oxygen (P = 0.157), when grouped by sample type. Inlet samples had greater colored dissolved organic matter, turbidity, nitrate plus nitrite N, and chlorophyll-a concentrations, and lower salinity than all other sample types (P< 0.0001 for all parameters). Outfall samples had significantly greater concentrations of nitrogen, total nitrogen, and total phosphorus (P < 0.0001). Water density also varied significantly among sample types (P = 0.001), with reef water and outfall samples having intermediate densities between those observed for open ocean and inlet samples.

As reported previously (26), PMMoV and HPyV were quantified at inlet and outfall sites in 2014 (Supplementary Table S1). Due to the low frequency of quantifiable results, HPyV data were treated as binary (presence/absence) data. Pepper mild mottle viruses were generally on the order of 10^4 gene copies L^{-1} at outfalls and 10^2 gene copies L^{-1} at inlet sites. Human polyomavirus was similarly detected more frequently at outfalls, with nearly two-thirds of samples positive at the Miami Central outfall, compared to only about one-third (38.9%) at the Miami North outfall, with 16.7 to 33.3% of inlet samples testing positive for HPyV.

Alpha diversity of prokaryotic community

Sequence analysis found a range of 119 to 3,943 OTUs among all samples, with a mean Good's coverage of 98.8 ± 0.8%. When measured by the Shannon index, samples collected from inlets had significantly lower alpha diversity (Table 2), than did the ocean and reef sites, while

the outfall and WWTP effluent samples had intermediate Shannon diversity values. Prokaryotic communities associated with corals (mucus and polyps) had significantly lower diversity than did all water samples. However, no significant differences ($P > 0.05$) were observed in ACE richness among sample types. Within a given sample type, differences in alpha diversity did not differ significantly by site, except among coral tissues, where ACE richness tended to increase at reef sites as follows: Barracuda = Emerald < Oakland Ridge < Pillars ($P = 0.034$, Tukey's *post-hoc* $P \geq 0.093$).

Prokaryotic community composition

Prokaryotic community composition was similar among all environmental water sample types (reef, open ocean water, inlet, and outfall water), with high relative abundances of *Alphaproteobacteria* and *Cyanobacteria* (Figure 2). In contrast, the WWTP effluent samples, before oceanic discharge, were predominantly comprised of *Betaproteobacteria*. Coral mucus and polyp samples had greater relative abundances of *Gammaproteobacteria*, with a greater relative abundance of *Bacilli* among polyps as compared to mucus samples. At higher taxonomic resolution, coral tissue communities primarily consisted of *Endozoicomonas* and *Bacillus*, with a relatively greater percentage of the community that could not be assigned to the genus level (Supplemental Figure S1). The percentage of *Endozoicomonas* was significantly different among tissue samples from each reef ($P = 0.038$), and tended to be higher at the Oakland Ridge reef. The abundances of *Bacillus* and *Paenibacillus* also differed significantly ($P = 0.008$ and 0.004, respectively) and tended to be greater among polyps collected from Barracuda and Emerald

reefs. Among all tissue samples, the percentage of unclassified reads was lower for polyp samples ($P < 0.0001$).

Beta diversity of prokaryotic communities

The bacterial prokaryotic composition, as evaluated by ANOSIM, did not differ significantly between open ocean and reef water sample types, when grouped by northern or southern sampling areas ($P = 0.911 – 0.914$). Prokaryotic communities in inlet and WWTP samples generally differed significantly from every other sample type ($P \leq 0.024$ and ≤ 0.005, respectively), except for the inlet and coral mucus samples collected from the southern area, which did not differ significantly ($P = 0.329$). Open ocean sites also did not differ from outfall samples in the northern region ($P = 0.104$), but community composition was significantly ($P = 0.015$) different among sampling sites in the southern sampling area. Reef communities differed from those of outfalls in either region ($P \leq 0.002$). Prokaryotic communities in coral mucus and polyp samples were generally significantly different from all the other sample types ($P \leq 0.009$), except as noted above. Communities from mucus and polyps did not differ from each other in the northern sampling region ($P = 0.225$), but they were significantly different among samples collected in the southern region ($P = 0.001$). Samples from different sites of the same sample type, did not harbor significantly different prokaryotic communities ($P \geq 0.119$).

Ordination of Bray-Curtis distances by principal coordinate analyses (Figure 3) revealed clustering by sample type, and this separation was supported by AMOVA ($P < 0.001$) for both sampling areas. Water samples tended to cluster together, apart from coral tissue and WWTP effluent samples. Similar to the ANOSIM results, in the southern region, *post-hoc* tests revealed

248 no significant separation of inlet from coral mucus samples (P = 0.225). Differences in sample
249 type were primarily attributable to the families *Cyanobacteria* GpIIa, *Flavobacteriaceae*,
250 *Candidatus Pelagibacter*, *Rhodospirillaceae*, and *Rhodobacteraceae* among water samples, and
251 these families were less abundant in outfall samples (Supplementary Figure S1). The WWTP
252 samples showed taxonomic variability between sampling areas, with the WWTP samples
253 associated with the northern region (Miami North) primarily comprised of *Burkholderiaceae*,
254 while those associated with the southern region (Miami Central) were predominantly comprised
255 of a large number of less abundant families. Coral tissue samples had significantly greater
256 abundances of *Bacillaceae* 1 and *Hahellaceae*.

257 Redundancy analysis generally supported the results of Kruskal-Wallis test (Figures S2
258 and S3). Coral tissue samples were more strongly associated with increased abundances of
259 *Bacillaceae* and *Hahellaceae* while inlet samples were associated with greater abundances of
260 *Flavobacteriaceae* and *Rhodobacteraceae*. Abundances of *Cyanobacteria* group II were also
261 associated with reef water samples. Little separation was observed between open ocean and
262 outfall samples, which clustered with ubiquitous marine families. Of note, water samples from
263 the Pillars reef site were more closely related to inlet samples than were samples from other
264 reefs.

265

266 **Fungal community diversity and composition**

267 Fungal community coverage was estimated at 97.9 ± 1.5% among all samples, with
268 between 62 to 1,125 OTUs in individual samples. Differences in alpha diversity among fungal
269 communities (Table 3) generally corresponded to those observed for prokaryotic communities

and are described in detail in Supplementary Results. A large portion of the fungal community among all samples could not be assigned to a phylum (Figure 4). Among the genera that could be classified, *Dictyocatenulata* and *Bullera* were predominantly found in water and WWTP samples, while *Dictyocatenulata* was among the only genus that could be classified from coral tissue samples. *Aspergillus* was also somewhat abundant among samples collected in the northern region, especially in ocean and reef water samples.

Differences in beta diversity among fungal communities from LBSP (*i.e.,* inlets and outfalls), reef water, and open ocean samples were similar to those described for prokaryotic communities (described in detail in Supplementary Results). In both northern and southern sampling areas, coral mucus and polyp communities differed significantly in community composition from those of other sample types ($P \leq 0.006$ and ≤ 0.040, with respect to sampling area), but communities characterized from mucus and polyps were not significantly different ($P = 0.478$ and 0.091, respectively). In both sampling regions, no differences in beta diversity were observed among samples of the same type between sampling sites ($P \geq 0.113$). Ordination of samples by PCoA showed poor correlations to distance matrices ($r^2 \leq 0.113$) and did not show any trends in sample clustering (data not shown).

Influence of land-based sources of pollution

Physicochemical parameters were presumed to vary primarily as a result of inputs from LBSP associated with changes between wet and dry seasons (46), with the exception of temperature. In order to determine which physicochemical factors and sampling locations best explained variation in phylogenetic structure, redundancy analysis relating sampling site

292 (independent variables, n=10), physiochemical parameters (independent variables, n=11), and
293 the relative abundance of bacterial families (dependent variable, n=15) among water samples
294 was conducted (Figure 5). The abundances of only a few prokaryotic families were related to
295 physicochemical parameters, while the majority clustered near the origin. Based on position in
296 the same quadrant of the RDA, salinity appeared to be positively related with abundances of
297 *Candidatus Pelagibacter*, *Oceanospirillaceae*, and *Rhodospirillaceae,* and negatively associated
298 with *Flavobacteriaceae* and *Rhodobacteraceae*. The abundances of *Cyanobacteria* group II were
299 also positively associated with water temperature, based on similar direction and positioning in
300 the RDA. Similar to ANOVA results (described above), the Miami Central outfall was
301 associated with increased nutrient concentrations, water density, and dissolved oxygen, while
302 inlet samples were associated with higher concentrations of nitrate plus nitrite N, chlorophyll-a,
303 turbidity, and CDOM

304 Binary logistic regression analysis revealed that prokaryotic community composition,
305 determined using abundant families, was not significantly) related to HPyV detection ($P \geq$
306 0.998), determined previously (26). However, the abundances of *Bacillaceae* and
307 *Methanomassiliicococcaceae* were significantly positively correlated with the concentration of
308 PMMoV (r = 0.366 and 0.413, P = 0.015 and 0.006). In contrast, abundances of
309 *Flavobacteriaceae*, *Rhodospirillaceae*, *Rhodobacteraceae*, and *Micromonasporaceae* were
310 negatively correlated with PMMoV concentrations (r = -0.335 to -0.708, $P \leq$ 0.027).

311

312 **Source contributions**

313 Evaluation of prokaryotic source contributions using the SourceTracker sequence
314 analysis software revealed that communities were predominantly comprised of bacteria
315 ubiquitous to marine samples, as well as host-specific OTUs among coral tissue samples (Figure
316 6A). Among the LBSPs, outfall communities had the greatest influence on the designated
317 sources on community composition among reef water and mucus samples, followed by the
318 influence of communities from inlet samples. Among reef water samples, the contribution from
319 outfall communities was significantly greater at sites collected from the southern region than
320 others (Emerald and Pillars reefs, $P = 0.007$), although other source contributions did not differ
321 significantly by site ($P \geq 0.052$). The contribution of bacteria from source communities to those
322 of reef water and polyps also showed temporal variability (Table S2) and were generally greater
323 in samples collected in 2014 than those in 2015. Mucus communities did not show temporal
324 variability in source influence ($P \geq 0.553$, Table S2).

325 Fungal communities showed a lower proportion of ubiquitous marine taxa (open ocean
326 water source) than did bacterial communities (Figure 6B). The outfall contribution was
327 predominant among reef water samples, with some influence from inlet communities, while coral
328 mucus and polyp samples were predominantly comprised of host-specific OTUs. Reef water
329 samples collected in the northern region (Oakland Ridge and Barracuda reefs) had significantly
330 higher percentages of the fungal communities associated with those from open ocean samples (P
331 $= 0.012$), while reef communities in the southern region had significantly greater proportions of
332 the community from unknown sources ($P = 0.004$). Similar to bacterial communities, no
333 difference in source contributions by site was observed among mucus samples ($P \geq 0.083$),
334 although polyp communities differed significantly by site for all source categories ($P \leq 0.046$),

except that from WWTP ($P = 0.114$). Fungal source contributions showed less temporal variation than did those from prokaryotic communities (Table S3). Fungal contributions from inlets to reef water communities were greater in 2014, while outfall contributions to reef communities tended to be greater from May to Nov in both 2014 and 2015. Fungal contributions from outfall communities to polyp communities also tended to be higher in 2014.

DISCUSSION

The prokaryotic communities characterized in this study using Illumina-based sequence technology were similar to those previously characterized in marine coastal waters in this area using 454 pyrosequencing technology (25). The marine water communities were primarily comprised of *Proteobacteria* and *Cyanobacteria*, and members of the families *Flavobacteriaceae* and *Rhodobacteriaceae* were found to vary in abundance between inlet and ocean/reef samples due, in part, to changes in salinity.

Communities from coral tissue samples showed some variation from previous reports (4, 5, 12). While *Alpha-* and *Gamma-Proteobacteria* as well as *Cyanobacteria* were the most abundant phyla, members of the *Bacteroidetes* and *Firmicutes*, previously reported to be associated with *Porites astreoides* (12), these were detected here at low abundances. Instead, members of the class *Bacilli,* including the genera *Bacillius* and *Paenibacillus,* were abundant. Furthermore, the genus *Endozoicomonas*, previously reported to be an important member of a core coral microbiome (4), was abundant from samples at only at the Oakland Ridge reef, lying farthest north in the sampling area. Differences in taxonomic composition among studies may be

357 due to characterization method, including differences in primers used for next-generation
358 sequencing (47), as well as geographic and physicochemical differences affecting coral
359 communities (13).

360 Fungal communities in open ocean environments are only beginning to receive the same
361 attention as prokaryotes and remain largely uncharacterized (48). While the majority of fungal
362 community sequences were unclassified in this study, a previous report found members of class
363 *Sordariomycetes* to be abundant in *Porites astreoides* near Panama (5). Among genera classified,
364 *Bullera* has recently been reported from marine seafloor sediment (49), but much less is known
365 about the genus *Dictyocatenulata*, a stilbellacaeous fungal group associated with bark and wood
366 (50), which was among the only genus classified among coral samples. Fungal results, especially
367 taxonomic classifications, presented here should be considered cautiously due to a number of
368 issues that have recently received attention regarding the use of next-generation sequencing for
369 ecological studies of fungi, including lack of a control mock community, sequencing target,
370 alignment and clustering methods, and incomplete taxonomic databases (51, 52). Nevertheless,
371 OTU-based analyses tended to agree well with those from prokaryotic community
372 characterization (discussed below), suggesting that unclassified sequences may simply represent
373 unexplored marine fungal diversity (48). Further analyses must await new sequencing
374 technologies, bioinformatics approaches, and further development of taxonomic databases.
375 Culture-based approaches may also benefit these analyses.

376 The prokaryotic and fungal communities were shown to differ significantly as a result of
377 sample type (*i.e.* ocean, reef water, WWTP outfalls, and inlets). A previous study also
378 characterized prokaryotic communities from ocean, reef, outfall, and inlet samples off the
379 southeastern Florida coast and similarly found significant differences in the phylogenetic

structures of communities associated with different sample types (25). This prior study, done using 454 technology, also noted that differences in community composition were associated with seasonal changes in rainfall (25). Seasonal variation of bacterial communities in coastal marine waters have been well established using next-generation sequencing approaches, with shifts in community structure associated with temperature, daylight, rainfall, and nutrient concentrations (25, 53). While seasonal changes in rainfall likely effect the magnitude of inputs from LBSP (46), this variation was presumed to affect sampling sites similarly in the current study.

Importantly, the differences in prokaryotic communities among types of water samples varied based on the area of the study region sampled. In the northern area, no differences were observed between outfall and ocean communities, suggesting minimal impacts from treated wastewater. However, this was not the case in the southern region, where the outfall communities differed significantly from those in both ocean and reef samples. Similarly, coral mucus communities could not be significantly differentiated from inlet communities in the southern region, but were distinct from those of water samples in the northern area. Analyses of fungi generally agreed with prokaryotic results, although outfall samples differed from ocean samples in the northern area, and reef fungal communities were not significantly different from fungal communities among samples collected in the southern area. Taken together, these results suggest that there is a greater amount of anthropogenic input from both treated wastewater and LBSP from inlets into the southern area, as well as potentially different distribution dynamics between bacterial and fungal communities.

SourceTracker analysis (40) revealed that outfall communities had a greater influence on those in reef water than did inlet communities, among both among prokaryotes and fungi. This

result is not surprising given the greater similarity in physicochemical parameters between reef and outfall samples *versus* reef and inlet chemistries. It should also be noted that differences in source contribution may also be affected by salinity, temperature, oxygen, and nutrient gradients, which have previously been shown to influence the composition of both prokaryotic and fungal communities (48, 54, 55). Therefore, influence from outfall communities may possibly be exaggerated to some extent due to species sorting dynamics rather than community exchange. Not surprisingly, communities from coral mucus showed greater susceptibility to source influence than did the polyp communities, most likely due to a more intimate association of the coral mucus with the surrounding waters (56). Among prokaryotic communities, those of coral polyps at the Pillars reef site showed the greatest influence from WWTP, and both sites sampled in the southern region (*i.e*. Emerald and Pillars Reefs) showed higher fungal contributions from outfall. Taken together with the overall prokaryotic community analyses, these data suggest that anthropogenic sources have a greater impact on coral communities at the Emerald and Pillars reef sites than at Oakland Ridge and Barracuda reefs. Furthermore, viral microbial source tracking marker concentrations were more frequently detected (HPyV) and found at higher concentrations (PMMoV) from Miami Central outfall than Miami North suggesting a stronger influence of human fecal contamination in the southern sampling area.

In this study, it was found that microbial communities primarily from WWTP outfalls were the predominant influence from LBSP sources on communities in reef water and coral tissues. Furthermore, temporal variation was observed in source contributions, as expected based on seasonal dynamics (46), but geographic variation was also observed and corresponded to previously reported differences in human microbial source tracking markers (26). Previous studies have shown that the WWTP discharge plumes from these oceanic outfalls tend to rise to

426 the surface, where they dilute out within a few kilometers of the outfalls and typically do not
427 descend to the reefs themselves (57). However, examination of microbial community exchanges
428 presented here suggest that microbial contaminants can and do reach the actual reef corals and
429 may influence the community structure of reef microbiota (and thus presumably influence the
430 health status and resiliency of reef ecosystems). While runoff contamination from LBSP has
431 been well-characterized (18), this study highlights the importance of the contribution of WWTP
432 outfalls to reef contamination.

433 As corals face ever increasing environmental stressors in an era of climate change, the
434 reduction of individual stressors that can be mediated, such as LBSP exposures, become ever
435 more critical. Future research will be necessary to better inform how these variations in coral and
436 coastal water microbial community structure influence the progression of diseases in order to
437 better protect these dynamic ecosystems.

438

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FIGURE LEGENDS

Figure 1 – Map of sites sampled. Symbols: green = ocean, blue = reef, pink = outfall, and red= inlet.

Figure 2 – Distribution of abundant bacterial classes among sample types collected from the (A) northern and (B) southern cruise regions.

Figure 3 – Principal coordinate analyses of bacterial communities collected from the (A) northern ($r^2 = 0.818$) and (B) southern ($r^2 = 0.787$) sampling regions.

Figure 4 – Distribution of fungal genera in samples collected from the northern (A) and southern (B) sampling regions.

Figure 5 – Redundancy analysis relating physicochemical parameters, sample site locations, and distributions of the 15 most abundant bacterial families among water samples. Families that clustered around the origin are not shown for clarity. Colors denote sample type: purple = reef water; blue = open ocean; black = inlets; and gray = outfalls.

Figure 6 – Percent of (A) bacterial community composition and (B) fungal community composition attributable to source communities as determined by SourceTracker.

Table 1. Physicochemical parameters measured among water samples*.

Sample Type	n	Salinity (‰)	Temperature (°C)	Density (Kg m^{-3})	CDOM (µg L^{-1})	Turbidity (NTU)	DO (mg L^{-1})	N+N (µM)	TKN (µM)	TN (µM)	TP (µM)	Chl-a (µg L^{-1})
Open Ocean	27	36.2 ± 0.2†	25.7 ± 2.0	24.0 ± 0.6	1.1 ± 0.8	1.3 ± 0.9	6.6 ± 0.2	0.3 ± 0.0	3.9 ± 1.8	4.2 ± 1.8	0.3 ± 0.1	0.4 ± 0.2
Inlet	25	35.1 ± 1.5	27.1 ± 2.7	22.7 ± 1.5	11.0 ± 11.5	4.1 ± 2.4	6.4 ± 0.3	1.0 ± 0.9	6.6 ± 3.5	7.6 ± 3.9	0.3 ± 0.1	1.0 ± 0.5
Reef Water	157	35.9 ± 0.9	26.5 ± 2.2	23.5 ± 1.2	1.6 ± 3.2	1.4 ± 1.5	6.5 ± 0.3	0.4 ± 0.3	4.1 ± 1.8	4.5 ± 1.8	0.2 ± 0.1	0.4 ± 0.3
Outfall	55	35.7 ± 1.0	26.8 ± 2.1	23.3 ± 1.2	2.2 ± 2.2	1.2 ± 0.6	6.5 ± 0.3	0.5 ± 0.5	11.2 ± 16.2	11.7 ± 16.3	0.5 ± 0.5	0.4 ± 0.2

*Values are means ± standard deviation among all samples.

†Parameters measured include salinity, temperature, density, colored dissolved organic matter (CDOM), turbidity, dissolved oxygen (DO), nitrate + nitrite (N+N), nitrogen (TKN), total nitrogen (TN), total phosphorus (TP), and chlorophyll a (Chl-a).

Table 2. Coverage and alpha diversity indices for bacterial communities among all samples collected, consolidated by sample type[*].

Type	n	Coverage (%)	S_{obs}[†]	Shannon[‡]	ACE
Open Ocean	25	98.7 ± 0.4	1095 ± 208	4.42 ± 0.22^{A}	2243 ± 804
Inlet	27	98.9 ± 0.7	765 ± 396	$3.57 \pm 0.67^{B,C}$	2070 ± 1386
Reef Water	147	98.8 ± 0.6	968 ± 395	4.19 ± 0.60^{A}	2001 ± 1144
Outfall	53	99.0 ± 0.7	875 ± 541	$3.99 \pm 0.79^{A,B}$	1774 ± 1180
WWTP	9	99.0 ± 0.9	821 ± 643	$3.66 \pm 1.10^{A,B,C}$	1765 ± 1569
Coral Tissue	87	98.7 ± 1.1	1184 ± 838	3.31 ± 1.39^{C}	1999 ± 1629
			P-value	<0.0001	0.734

[*]Values are means ± standard deviation among all samples.

[†]S_{obs}: number of OTUs observed.

[‡]Sample groups sharing the same superscript letter did not differ significantly in alpha diversity by Tukey's *post-hoc* test ($P > 0.05$).

Table 3- Coverage and alpha diversity indices for fungal communities among all samples collected, consolidated by sample type[*].

Sample Type	n	Coverage (%)	S_{obs}[†]	Index	
				Shannon[‡]	ACE
Open Ocean	17	97.1 ± 1.1	614 ± 238	$3.74 \pm 1.08^{A,B}$	1457 ± 560^{A}
Inlet	24	96.6 ± 1.0	630 ± 184	3.27 ± 0.95^{B}	$2012 \pm 759^{B,C}$
Reef Water	110	97.2 ± 1.1	558 ± 212	3.33 ± 1.02^{B}	1536 ± 624^{A}
Outfall	45	97.1 ± 1.1	540 ± 208	3.06 ± 1.10^{B}	$1691 \pm 665^{A,B}$
WWTP	10	96.0 ± 0.6	743 ± 125	4.33 ± 0.54^{A}	2376 ± 566^{C}
Coral Tissue	117	99.5 ± 0.2	106 ± 27	1.26 ± 0.55^{C}	352 ± 180^{D}
			P-value	< 0.0001	< 0.0001

[*]Vales are mean ± standard deviation among all samples.

[†]S_{obs}: number of OTUs observed.

[‡]Sample groups sharing the same superscript did not differ significantly in alpha diversity by Tukey's *post-hoc* test ($P > 0.05$).

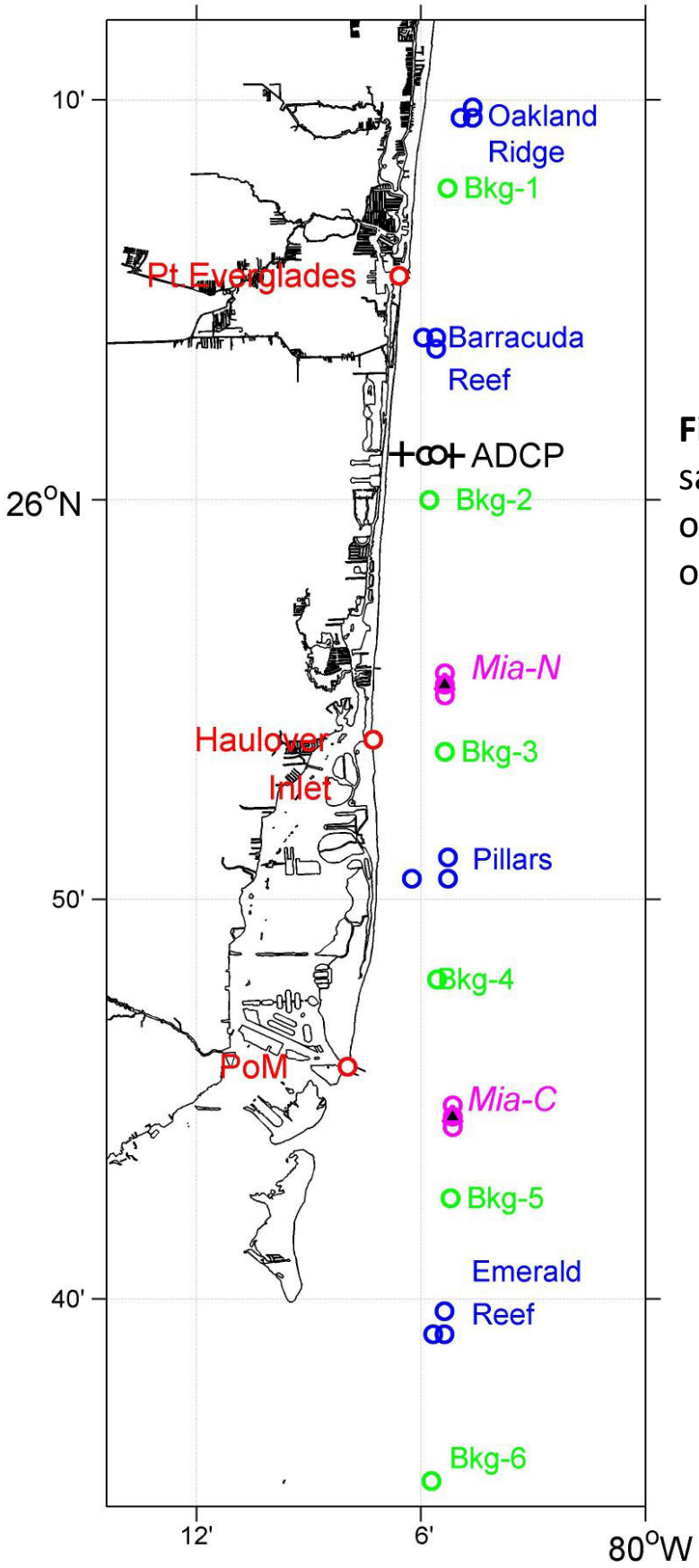

Figure 1 – Map of sites sampled. Symbols: green – ocean, blue – reef, pink – outfall, red – inlet.

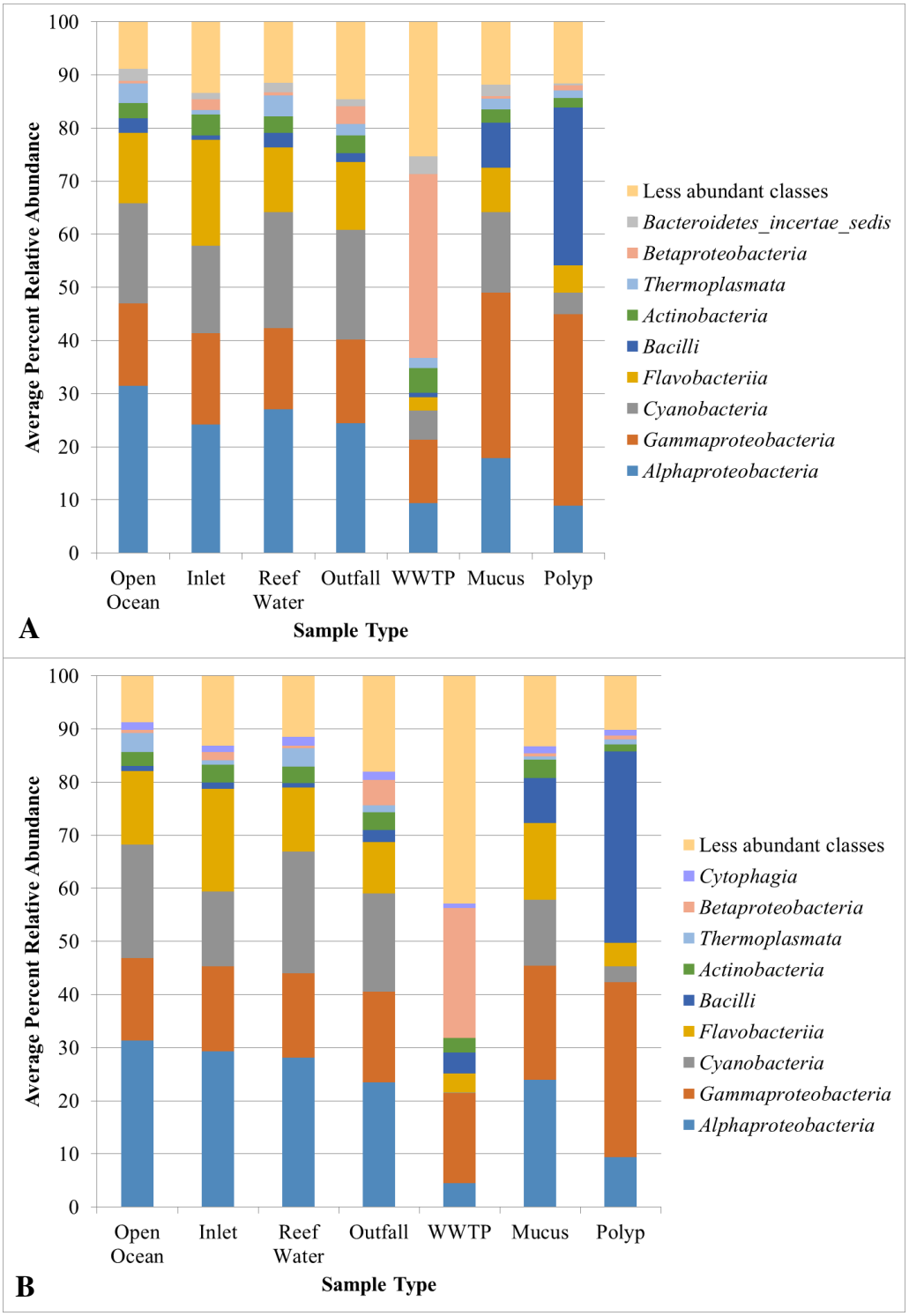

Figure 2 – Distribution of abundant bacterial classes among sample types collected from the northern (A) and southern (B) cruise regions.

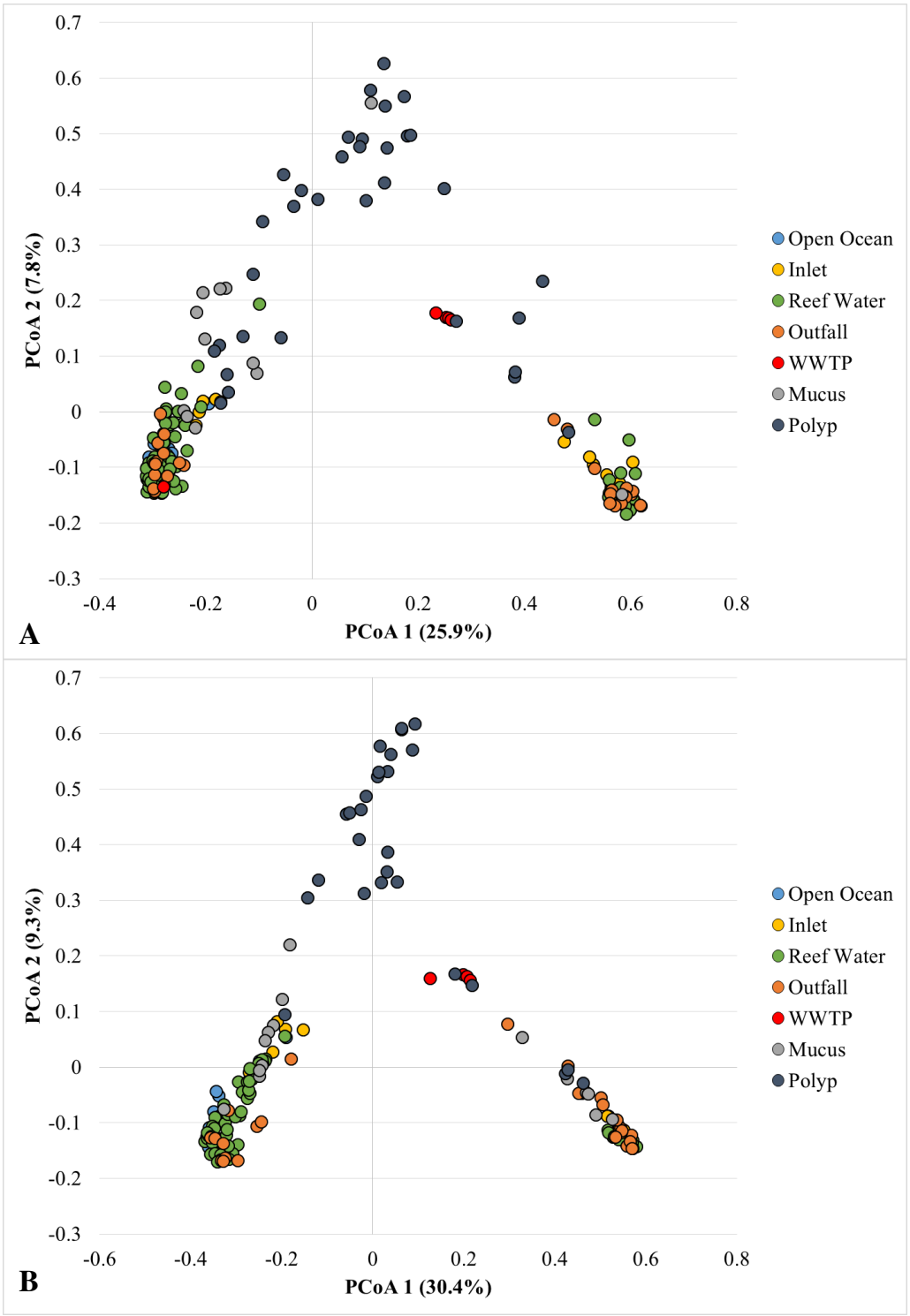

Figure 3 – Principal coordinate analyses of bacterial communities collected on from the northern (A, r^2 = 0.818) and southern (B, r^2 = 0.787) sampling regions.

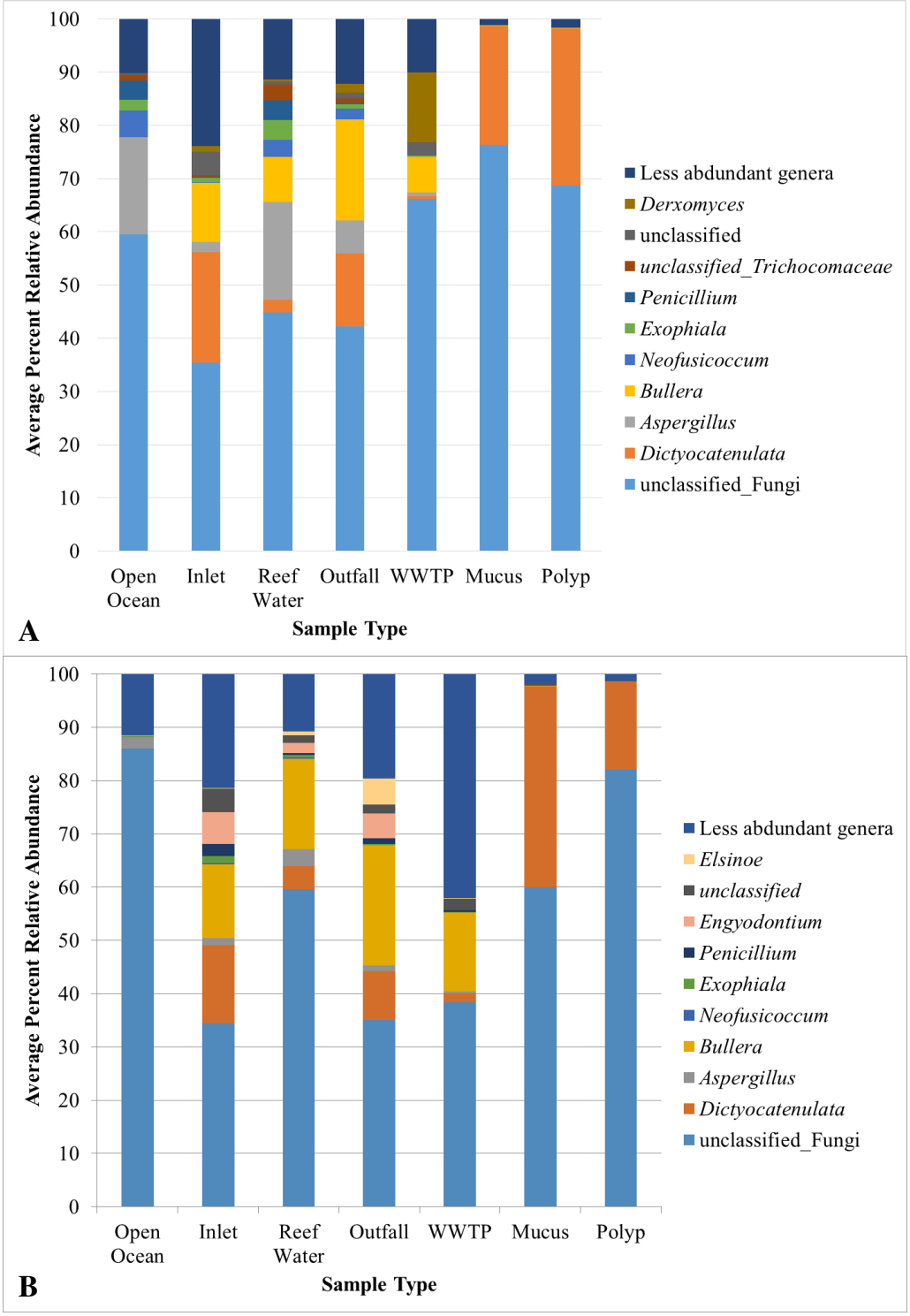

Figure 4 – Distribution of fungal genera in samples collected from the northern (A) and southern (B) sampling regions.

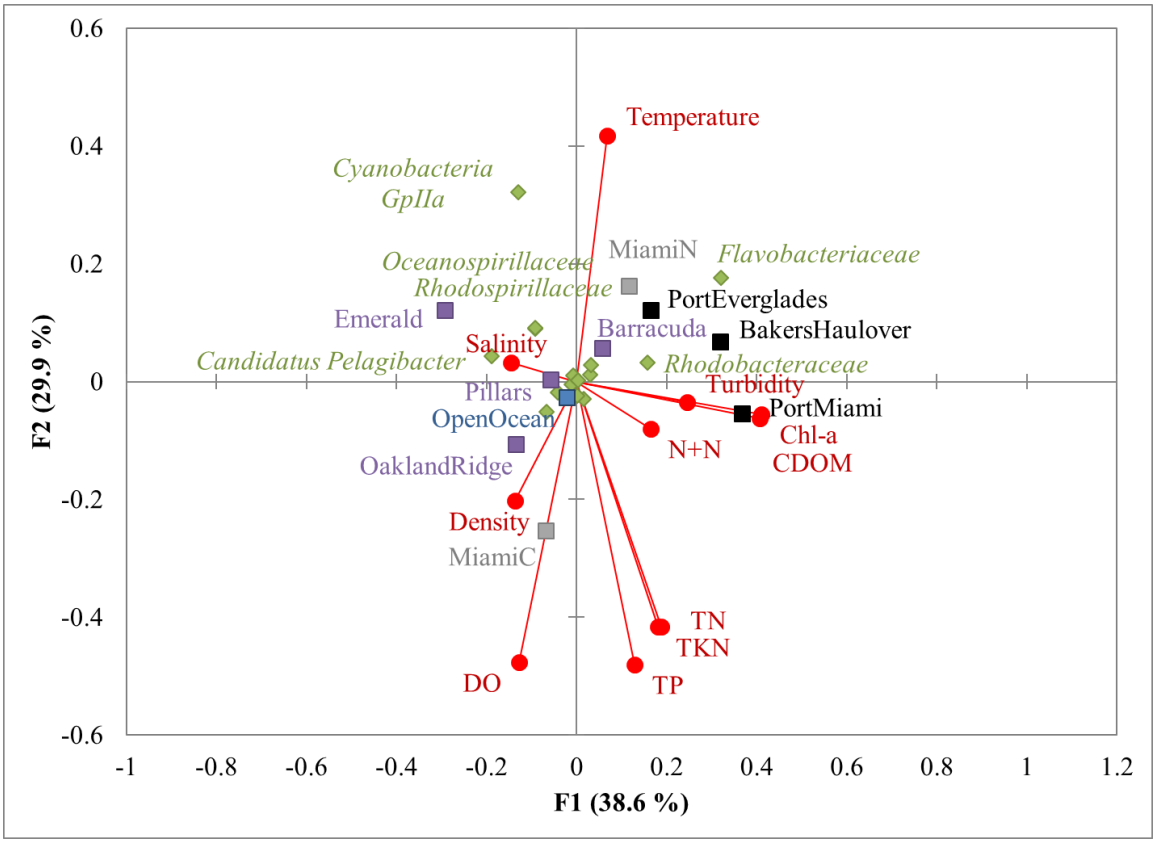

Figure 5 – Redundancy analysis relating physicochemical parameters, sample site locations, and distributions of the 15 most abundant bacterial families among water samples. For clarity, families that clustered around the origin are not shown. Colors denote sample type: purple – reef water; blue – open ocean; black – inlets; gray – outfalls.

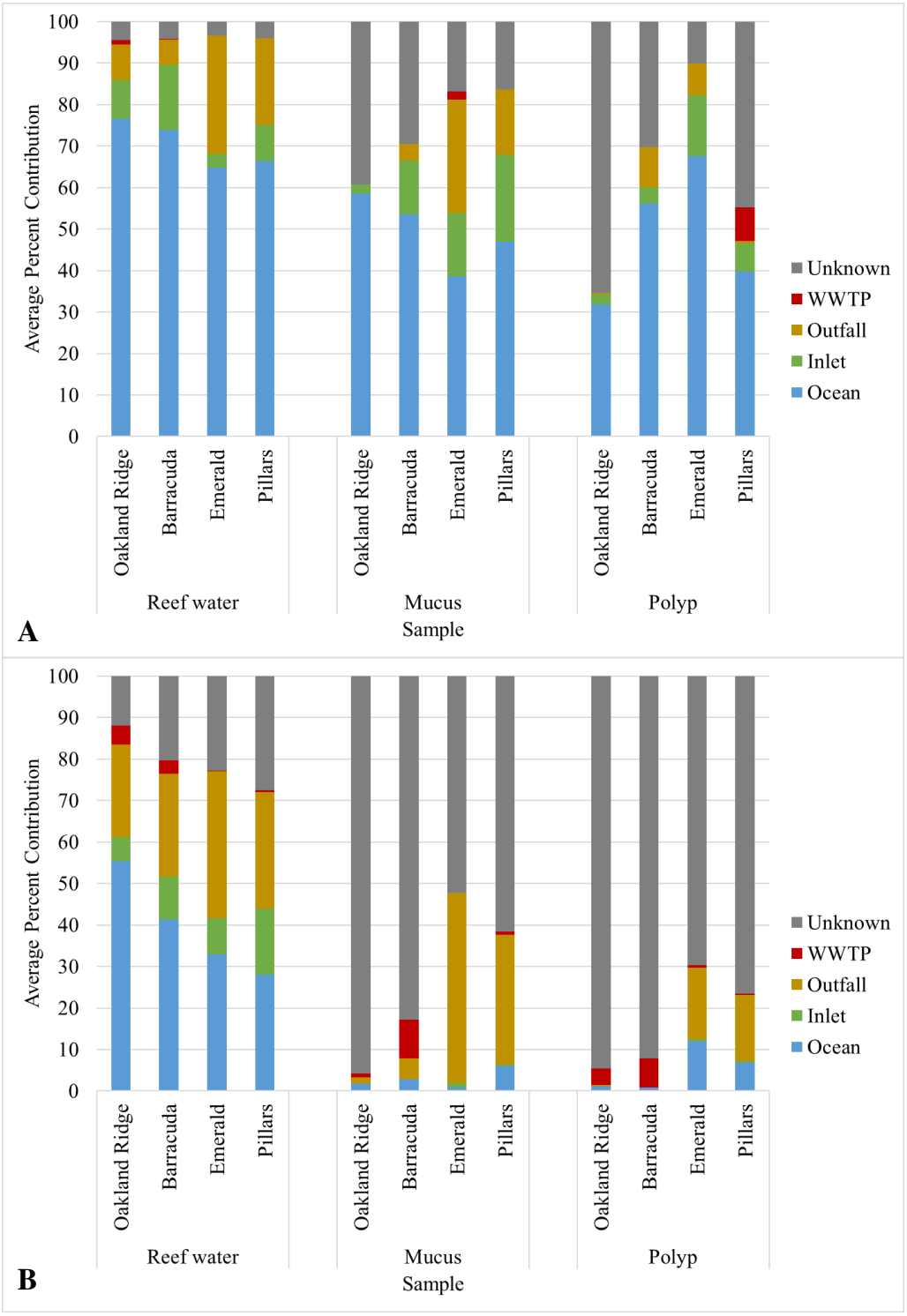

Figure 6 – Percent of (A) bacterial community composition and (B) fungal community composition attributable to source communities as determined by SourceTracker.